A Biosynthetic Strategy for Re-engineering the *Staphylococcus aureus* Cell Wall with Non-Native Small Molecules

Supporting Information

James W. Nelson,[1] Alexander G. Chamessian[1], Patrick J. McEnaney,[1] Ryan P. Murelli[1], Barbara I. Kazmierczak,[2] David A. Spiegel[1]

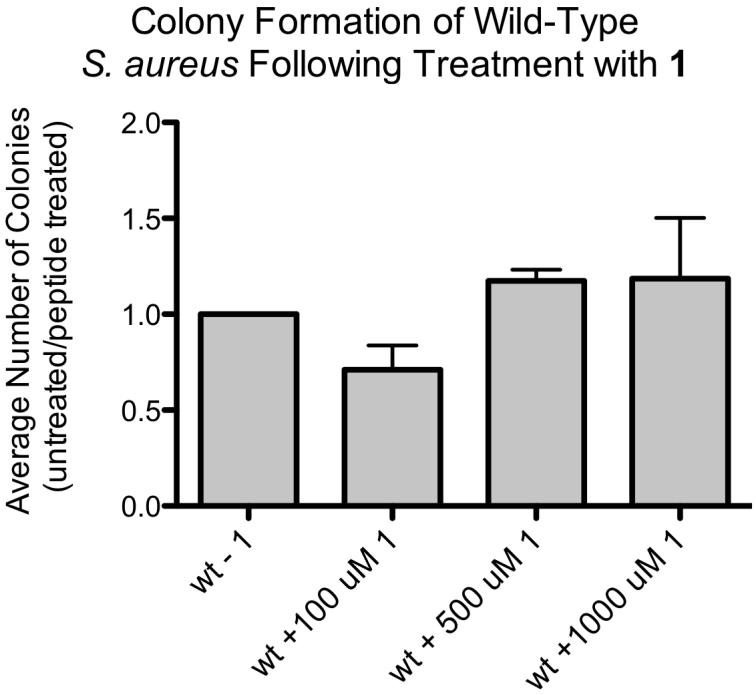
[1]*Department of Chemistry, Yale University, New Haven, Connecticut 06520*

[2]*Department of Medicine (Infectious Diseases), Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06520*

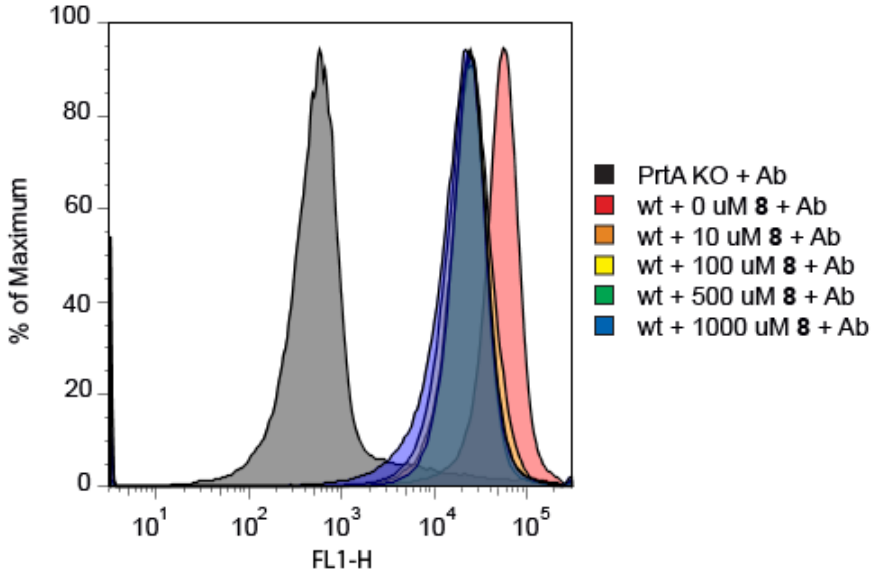
**corresponding author,* email: david.spiegel@yale.edu

Table of Contents

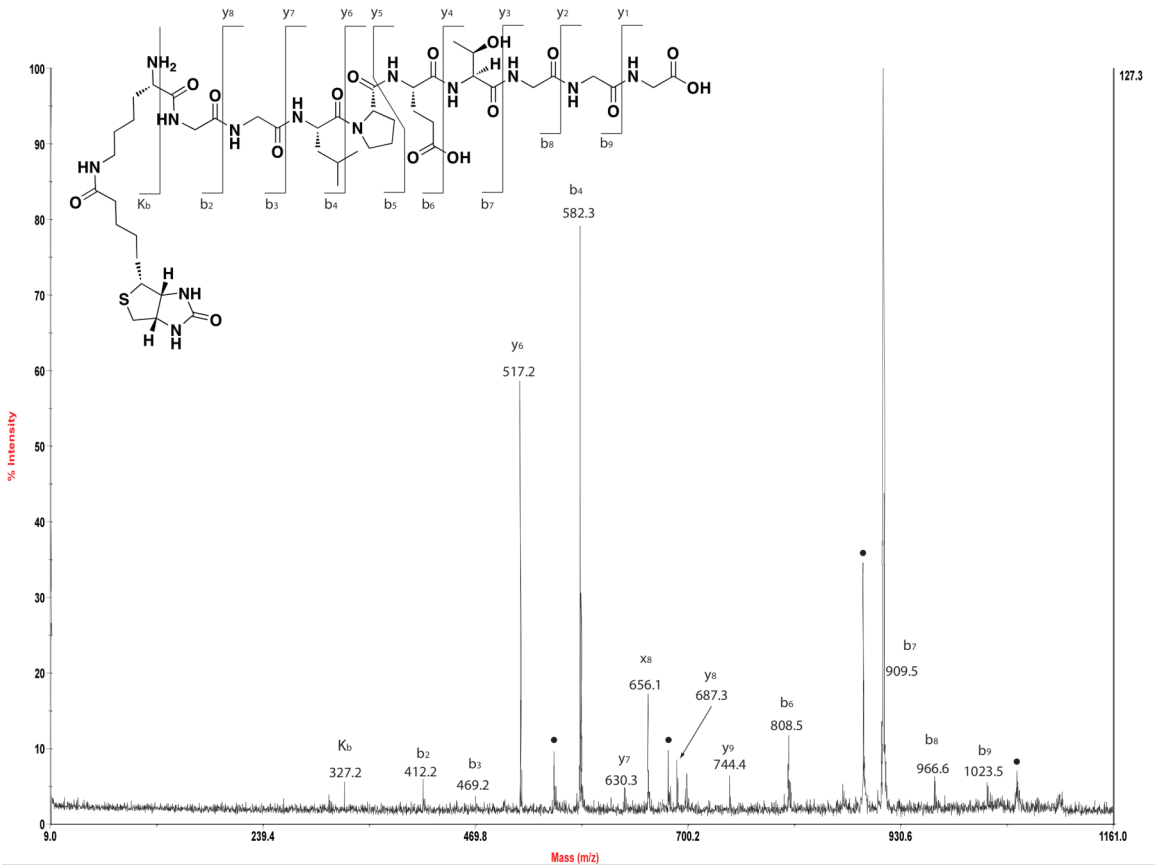
Supplementary Figures.



Supplementary Figure 1. Cell Viability Assessments. Performed as described below in the section labeled "Cell Viability Measurements." Abbreviations: "wt" indicated wild-type, and "peptide" indicates small molecule-peptide hybrid 1. Data are the average of triplicate experiments ± SEM.



Supplementary Figure 2. Effect of Treatment of Exogenous Sortase A Substrate on Endogenous Protein A Cell Surface Expression Levels. Cell-Surface Expression Levels of Protein A of **8**-treated S. aureus. Wild-type *S. aureus* (abbreviated "wt" in the Figure) and Protein–A knockout *S. aureus* (abbreviated "PrtA KO" in the Figure) were grown in the indicated concentrations of **8** for 24 hours at 37°C, washed three times with PBS, and incubated with 10 mg/mL rabbit anti-DNP IgG (abbreviated "Ab" in the Figure) conjugated to Alexa Fluor 488 for 1 hour at room temperature. Following another three washes, bacteria were examined by flow cytometry. Data presented are representative of multiple experiments.



Supplementary Figure 3. MS-MS Fragmentation of Isolated Bacterial Wall Fragments. The peak at 1098.6 was subjected to MS-MS fragmentation. The complete b-ion sequence, except b_1 and b_5 (unlikely to be observed due to the presence of proline) and the complete y-ion sequence N-terminal to the proline were observed. Unidentified peaks, which likely derive from a small amount of contaminant, are labeled with black dots.

Materials and Strains.

N^{α}-Fmoc-N^{ε}-Lys(5- and 6-carboxyfluorescein)-OH (mixture of isomers) and N^{α}-Fmoc-N^{ε}-Lys(Biotin)-OH were purchased from Anaspec (San Jose,

CA). All other amino acids were purchased from EMD Chemicals, Inc. (Philadelphia, PA). Rink Amide MBHA Resin preloaded with either Gly or Pro was purchased from Peptides International (Louisville, KY). HBTU was obtained from American Bioanalytical (Natick, MA). All solvents and reagents were used without further purification unless otherwise indicated. Lysostaphin (EC 3.4.24.75), obtained as a lyophilized powder from *Staphylococcus staphylolyticus*, and mutanolysin, isolated from *Streptomyces globisprus* ATCC 21553, were both obtained from Sigma Aldrich (St. Louis, MO). Rabbit anti-DNP IgG conjugated to AlexaFluor 488 were purchased from Invitrogen Corporation (Life Technologies, Carlsbad, CA).

Luria Broth (LB) plates were prepared by mixing 10g NaCl (Mallinckrodt Chemicals, Philipsburg, NJ), 10g tryptone (American Bioanalytical), 5g yeast extract (USB Corporation, Cleveland, OH), and 15g agar (American Bioanalytical) in 900 mL of distilled water. The mixture was autoclaved and diluted to 1L using water passed through a sterile filter (0.2 μm). LB Media was prepared in an identical fashion without agar. Phosphate-buffered saline (PBS) buffer was prepared as a 10x stock by mixing 800g NaCl, 20g KCL, 144g Na_2HPO_4, and 24g KH_2PO_4 (all from Mallinckrodt Chemicals) in 10L of deionized water and autoclaved. Sucrose-Maleate-$MgCl_2$ (SMM) buffer was prepared by adding 85.6 g sucrose (American Bioanalytical), 1.6 g maleate (Fisher Scientific), and 2.033 g $MgCl_2$ to 1 L of deionized water which was subsequently autoclaved. SMM and PBS buffers were freshly diluted prior to

use. Paraformaldehyde (PFA) was obtained from USB Corporation as a 4% solution in PBS and stored at 4°C.

Wild-type *S. aureus* strain (Newman)[1] was donated by Sheldon Campbell (Yale University Medical School, New Haven, CT). A *S. aureus* SrtA knockout strain in the Newman genetic background[2] was obtained from Olaf Schneewind (University of Chicago, Chicago, IL). *S. aureus* strain *spa::kan*,[3] a protein A knockout strain, was obtained from Timothy J. Foster (Trinity College, Dublin, Ireland).

Peptide Synthesis and Characterization

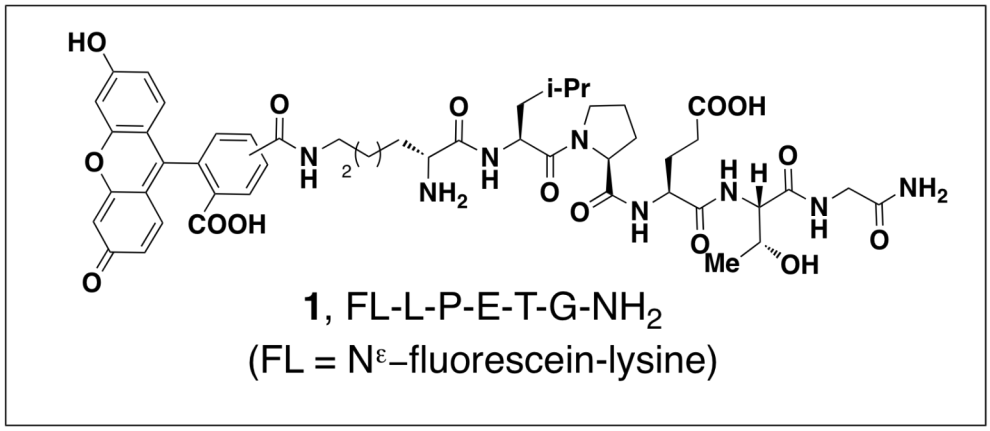
Rink amide resin (300 mg) preloaded with either N^a-Fmoc-Gly or N^a-Fmoc-Pro was mixed with 4 column volumes (CV) of dimethylformamide (DMF) for one hour prior to the commencement of each peptide synthesis. Deprotection of the resin prior to coupling was accomplished on shaking in 4 CV 20% piperidine/DMF for thirty minutes. For all subsequent coupling reactions, Fmoc-protected amino acid (5 equiv), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 5 equiv), and *i*-Pr_2NEt (10 equiv) were suspended with the resin in DMF (4 CV) and shaken at room temperature for a period of 4 hours on a Rotamix Shaker (Appropriate Technical Resources, Laurel, MD). In all syntheses, Fmoc-protecting groups were removed by shaking for five minutes in 50% piperidine/DMF (4 CV).

For peptide cleavage reactions, resin was first treated with a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (94:3:3, 10 mL) at room temperature for 90 minutes. Cleavage solutions were then filtered through cotton into cold ether, pelleted, resuspended twice in ether, and dried *in vacuo*. Peptides were purified via High Pressure Liquid Chromatography (HPLC) using a Dynamax Rainin Solvent Delivery System equipped with a Varian Prostar Detector (Galaxie Chromatography Data System version 1.8.505.5), and absorbance measurements were made at 214 and 254 nm simultaneously. A Waters Xterra Prep MS C18 7.8x150mm column was used for semi-preparative purifications using an acetonitrile:water gradient containing 0.1% TFA at 5.0 mL/min, as specified below for individual compounds. Purity was assessed *via* HPLC employing a Varian C8 4.6x250mm Microsorb C8 column run at a flow rate of 1.0 mL/min. Purified peptides were lyophilized and stored for subsequent use.

Peptides were analyzed via high-resolution mass spectroscopy obtained from a Bruker (Billerica, MA) 9.4 Tesla Apex-Qe Hybrid Qe-Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer consisting of an Apollo II electrospray ionization source. The sample was directly infused into the FT-ICR MS by nanoESI with a 30μm id fuse silica tip (New Objective, Inc.) at a 15μL/hr flow rate. The nESI tip was grounded and ~2100V potential was applied on the glass capillary endcap. The instrument (running Compass Software with APEX control acquisition component (v.1.2)) is setup to acquire single fid (512K) data with a mass range (m/z)

from 100 to 2000. Exact masses were obtained for the entire broadband spectrum. Subsequently, various instrument parameters were adjusted to maximize the signal(s) around the peak of interest. Bruker Daltonics DataAnalysis software (v. 3.4) was utilized for the analysis of the data and assignments were made based on exact mass measurements and fit of isotopic peaks to that of theoretical isotopic patterns (IsotopePattern algorithm, Bruker). Collected time transient data were zero filled, Fourier transformed, magnitude calculations to generated m/z mass spectrum.

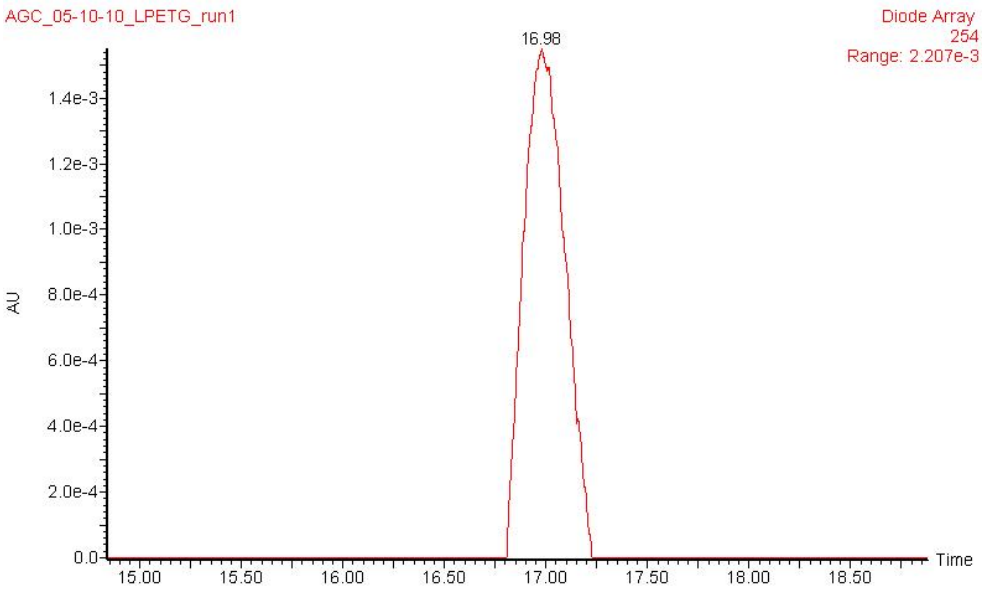
Spectral Data for Peptide 1.



1, FL-L-P-E-T-G-NH_2

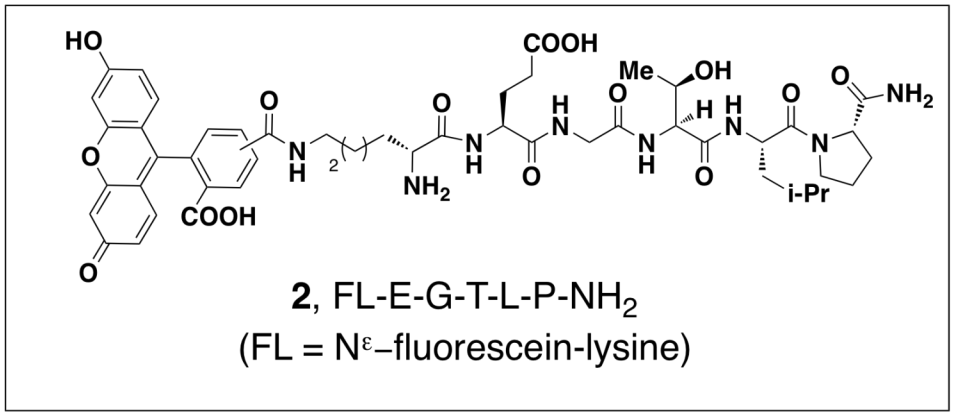
(FL = N^{ε}–fluorescein-lysine)

Fl-LPETG-NH_2 (**1**). Prepared using the general method for solid–phase peptide synthesis (above), and purified by HPLC (0–100% H_2O:ACN 60 minutes) after lyophilization as a white solid (100% purity, see Spectrum 1, below). HRMS (ES+) *m/z* 1001.4264 [calc'd for $C_{49}H_{60}N_8O_{15}$ (M+H) 1001.4251].



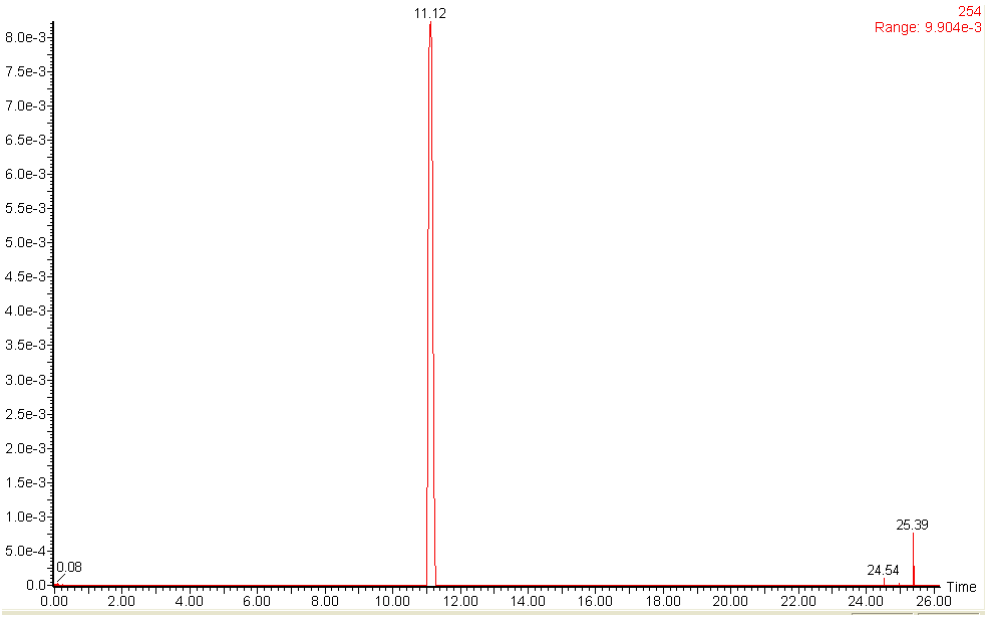
Spectrum 1. Analytical HPLC spectrum of compound **1** following purification.

Spectral Data for Peptide 2



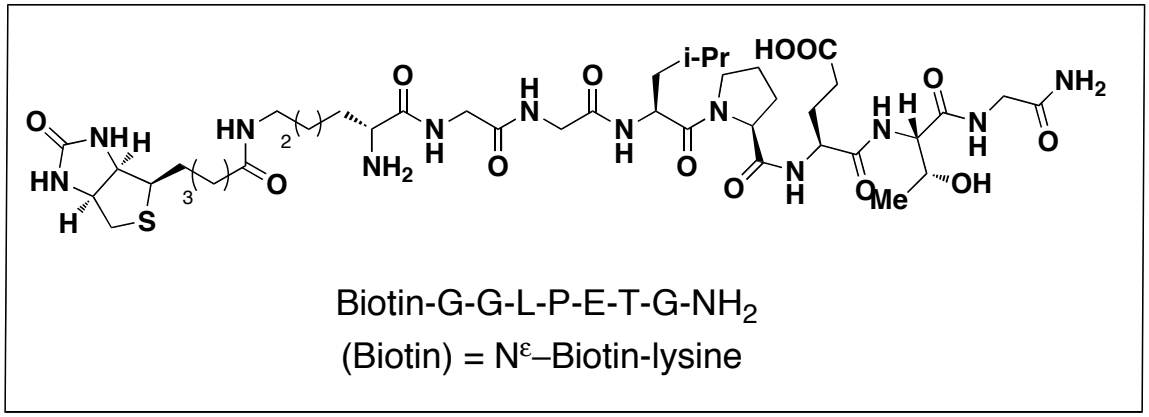
2, FL-E-G-T-L-P-NH_2
(FL = N^{ε}–fluorescein-lysine)

Fl-EGTLP-NH_2 (**2**). Prepared using the general method for solid–phase peptide synthesis (above), and purified by HPLC (0–100% H_2O:ACN 7.5 minutes). Isolated after lyophilization as a white solid (98% purity, see Spectrum 2, below). HRMS (ES+) *m/z* 1001.4273 [calc'd for $C_{48}H_{60}N_8O_{15}$ (M+H) 1001.4251].



Spectrum 2. Analytical HPLC trace for compound **2** following purification.

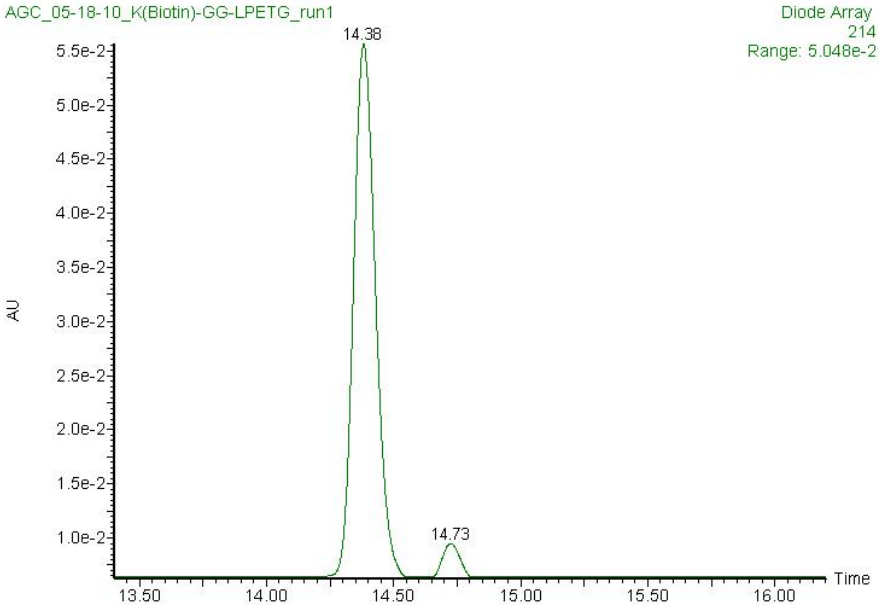
Spectral Data for Peptide 3



Biotin-G-G-L-P-E-T-G-NH_2

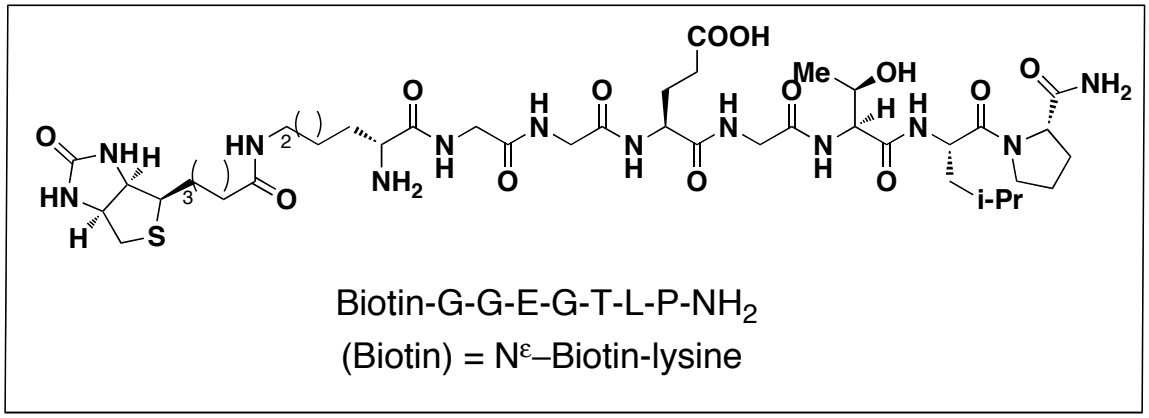
(Biotin) = N^{ε}–Biotin-lysine

Biotin-GG-LPETG-NH_2 (**3**). Prepared using the general method for solid–phase peptide synthesis (above) and purified by HPLC (0–40% H_2O:ACN 60 minutes). Peptide was isolated after lyophilization as a white solid (93.5% purity, see Spectrum 3, below). HRMS 983.496 *m/z* [calc'd for $C_{46}H_{79}N_{11}O_{15}S$ (M+H) 984.15].



Spectrum 3. Analytical HPLC trace for compound **3** following purification.

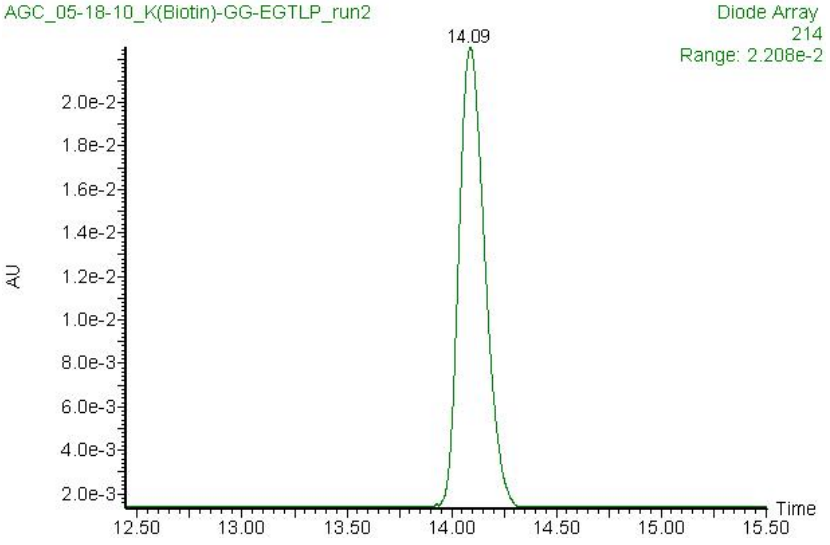
Spectral Data for Peptide 4



Biotin-G-G-E-G-T-L-P-NH_2

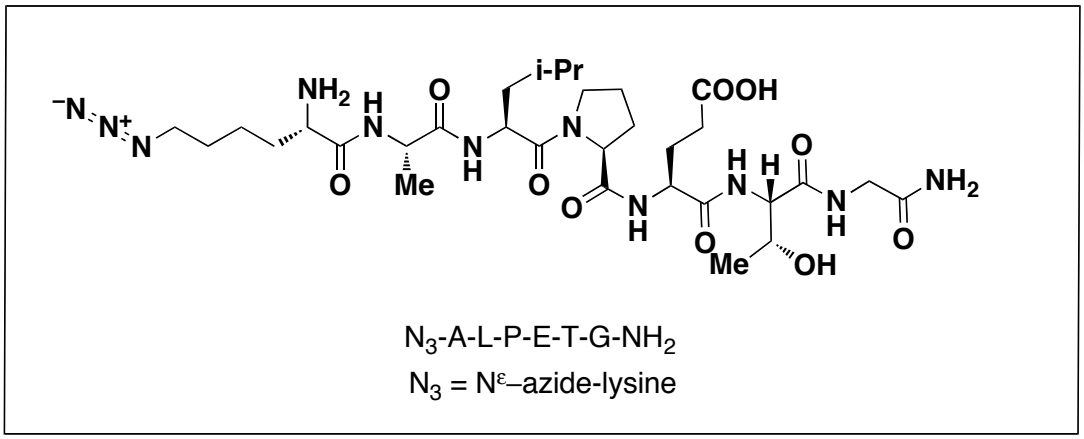
(Biotin) = N^{ε}–Biotin-lysine

Biotin-GG-EGTLP-NH_2 (**4**). Prepared using the general method for solid–phase peptide synthesis (above) and purified by HPLC (0–40% H_2O:ACN 60 minutes). Peptide was isolated after lyophilization as a white solid (100% purity, see Spectrum 3, below). HRMS 983.496 *m/z* [calc'd for $C_{46}H_{79}N_{11}O_{15}S$ (M+H) 984.15].



Spectrum 4. Analytical HPLC trace for compound **4** following purification.

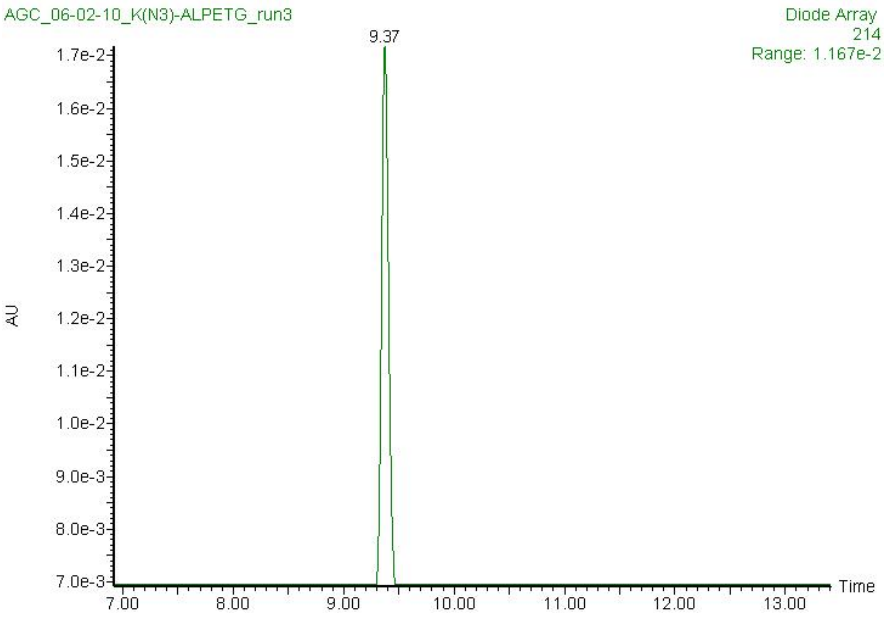
Spectral Data for Peptide 5



N_3-A-L-P-E-T-G-NH_2

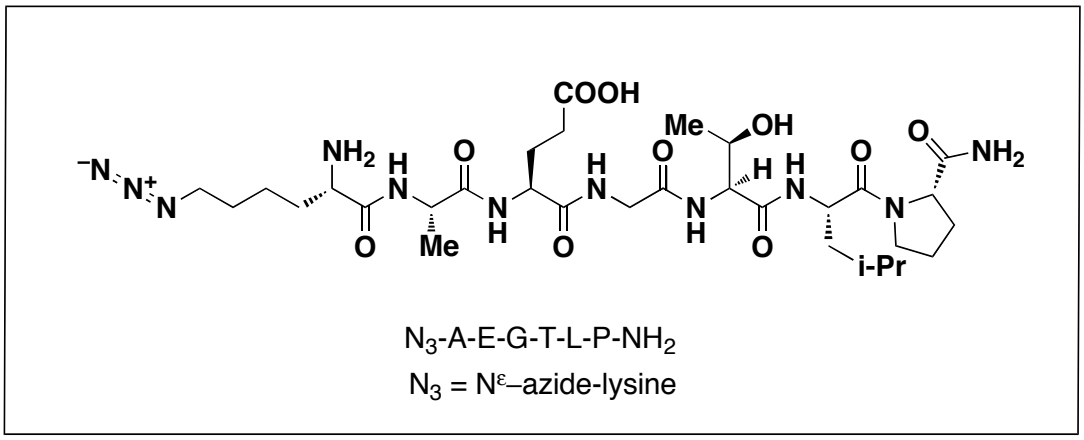
N_3 = N^{ε}–azide-lysine

N_3-ALPETG-NH_2 (**5**). Prepared using the general method for solid–phase peptide synthesis (above) and purified by HPLC (0–60% H_2O:ACN 60 minutes). Peptide was isolated after lyophilization as a white solid (100% purity, see Spectrum 5, below). HRMS 740.29 *m/z* [calc'd for $C_{31}H_{53}N_{11}O_{10}$ (M+H) 740.40].



Spectrum 5. Analytical HPLC trace for compound **5** following purification.

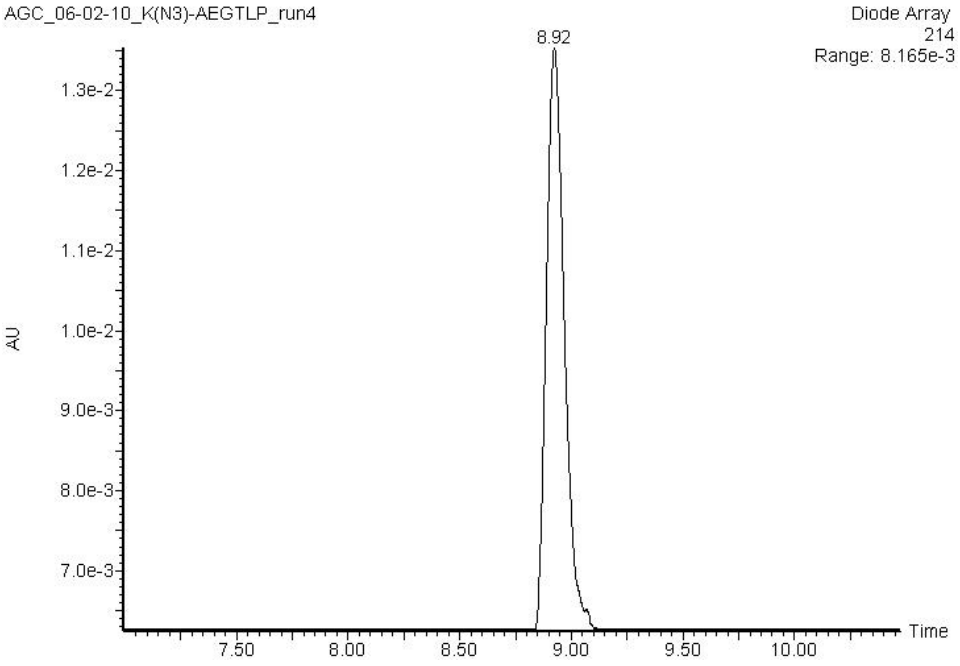
Spectral Data for Peptide 6



N_3-A-E-G-T-L-P-NH_2

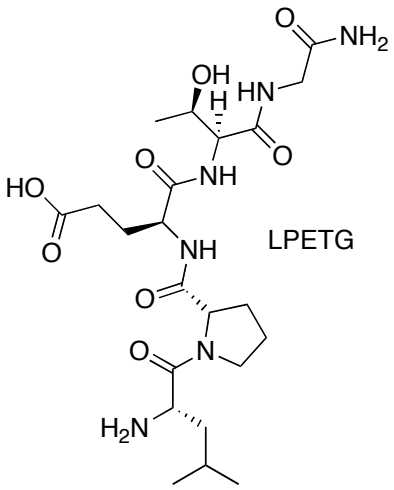
N_3 = N^{ε}–azide-lysine

N_3-AEGTLP-NH_2 (**5**). Prepared using the general method for solid–phase peptide synthesis (above) and purified by HPLC (0–60% H_2O:ACN 60 minutes). Peptide was isolated after lyophilization as a white solid (100% purity, see Spectrum 6, below). HRMS 740.31 *m/z* [calc'd for $C_{31}H_{53}N_{11}O_{10}$ (M+H) 740.40].

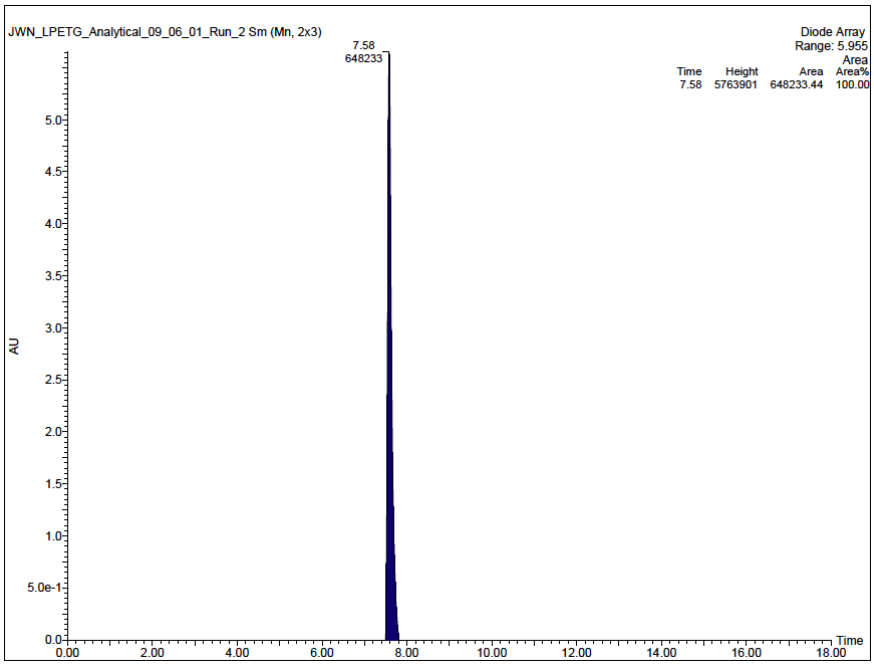


Spectrum 6. Analytical HPLC trace for compound **6** following purification.

Spectral Data for Peptide 8

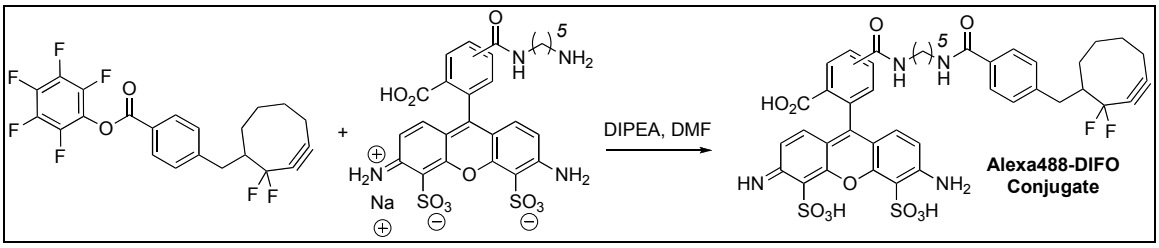


LPETG-NH_2 (**8**). Prepared using the general method for solid–phase peptide synthesis (above) and purified by HPLC (0–40% H_2O:ACN 40 minutes). Peptide was isolated after lyophilization as a white solid (100% purity, see Spectrum 8, below). HRMS (ES+) 515.28 (*m*+3) *m/z* [calc'd for $C_{46}H_{79}N_{11}O_{15}S$ (M+H) 515.28].

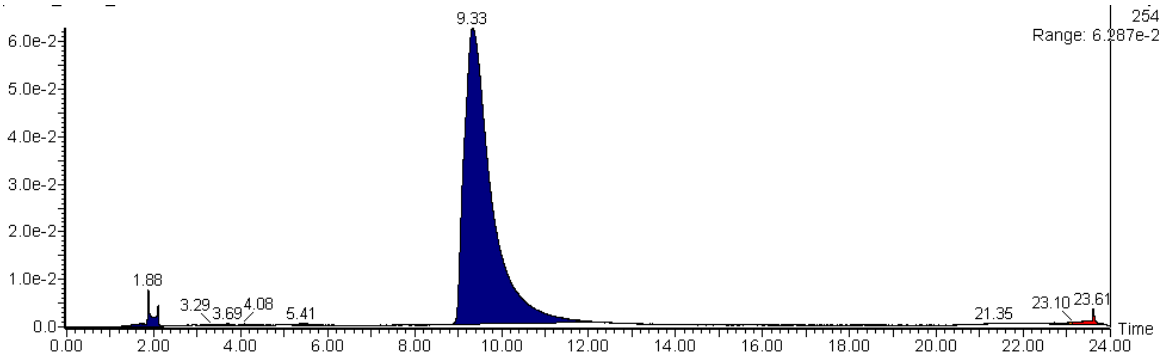


Spectrum 7 Analytical HPLC of **8** following purification.

Synthesis of Alexa Fluor 488-DIFO Conjugate (7)



Perfluorophenyl 4-((2,2-difluorocyclooct-3-ynyl)methyl)benzoate (3.1 mg, 7 mmol, 2 equiv.)[4] and Hunig's base (2.4 mL, 14 mmol, 4 equiv.) were dissolved in DMF (0.5 mL) followed by Alexa Fluor 488 cadaverine (2 mg, 3.1 mmol, 1 equiv.), whose transfer was aided by the use of DMF (0.5 mL). The reaction mixture stirred for 48 hours, and was concentrated and purified by HPLC ((Sunfire™ Prep C18 column [10 x 150 mm] using a 40% to 60% AcCN in H_2O gradient with 0.1% TFA over 24 min at 5 mL/min) to give **7**. Suspension in 2 mL of PBS led to a 200 mM solution (0.4 mmol, 13% yield) as determined by absorbance measurement along with the literature extinction coefficient of Alexa Fluor 488 of 71,000 $cm^{-1}M^{-1}$ at 495 nM wavelength.[5] (98% pure, see spectum **7**, below). HRMS (ES+) *m/z* 879.2112 [calc'd for $C_{42}H_{41}F_2N_4O_{11}S_2$ (M+1) 879.2181]



Spectrum 8 HPLC Spectrum of **7** following purification

Cell Viability Measurements

In order to determine the effect of small molecules on the viability of the Newman (wild-type) *S. aureus* strain, cultures grown in the presence and absence of peptide **1** at the indicated concentrations were serially diluted (10-fold), then plated on LB-plates. Colonies were counted from all plates on which individual colonies were distinguishable after overnight growth at 37 ^{o}C, and as shown (Supplementary Figure 1), no significant difference in growth was observed for cells incubated with up to 1.0 mM of **1**. Counts are represented as the normalized ratio of untreated cells to peptide treated cells.

Generic Protocol for Bacterial Growth and Labeling with Sortase Substrates

All media and materials were autoclaved prior to use unless otherwise stated. Bacteria were obtained in solid agar and grown on LB agar plates. Stocks of bacteria were kept at –80 °C in 25% sterile glycerol.

Bacteria were allowed to grow overnight at 37 °C on LB plates, then inoculated *via* flame-loop into LB media and allowed to grow again overnight (grown in an Innova 4200 Incubator, New Brunswick Scientific, New Brunswick, NJ). Aliquots of this culture were taken and added to LB media containing compounds at the desired concentrations (unless otherwise noted, bacteria were grown in 1 mM compound), and the cell count was normalized to an OD_{600} of 0.25. Compounds were dissolved in sterile water

prior to addition. Sortase-deficient mutants (*S. aureus* srtA$^-$)[2] were grown in the presence of 10 µg/mL erythromycin. Cultures were covered in aluminum foil to prevent possible photobleaching. Incubation of bacteria in LB was allowed to proceed for 24 hours at 37 °C. Subsequently, 100 µL aliquots of culture were taken, pelleted at 12000g for one minute, and washed three times with PBS buffer (using an Eppendorf 5417 centrifuge, Westbury, NY). Bacteria were then used in the following experiments.

Epifluorescence Microscopy and Flow Cytometry

After following the generic procedure for labeling above, cells were resuspended in 4% paraformaldehyde in PBS and fixed at room temperature on a nutating mixer for 45 minutes. Antibody staining (10 mg/mL) was carried out subsequently for indicated cell samples for one hour at room temperature. Following three additional PBS washes, bacteria were resuspended in 1 mL of PBS and analyzed on a Accuri C6 Flow Cytomer (Accuri Cytometers). 100 µL aliquots of the above were removed (prior to cytometric analysis), pelleted, and resuspended in 20 µL anti-fade media (Vectashield Mounting Media; Vector Laboratories), mounted onto Esco Superfrost® Microscope slides (Erie Scientific Company, Portsmouth NH), and allowed to incubate overnight at 4 °C before images were obtained.

Quantitation of Fluorescence via Cell-Wall Extract

Following the generic procedure above, bacteria were counted and volumes of bacteria labeled with **1** or **2** were taken such that equal amounts of bacteria were diluted to 3.5 mL with PBS, and subjected to ultrasonication using a Branson Sonifier 450 (three one minute sessions at 80% duty cycle, 4/10 output) equipped with a microtip. The sonicated cells were filtered via centrifugal filtration at 3600 rpm (2700 rcf) on a Sorvall Legend TR (Thermo Fisher Scientific, Waltham, MA) centrifuge using Amicon YM-10 filters (Millipore Corp., Billerica, MA) at 4 °C. The filtrate was taken and used to determine the fluorescence intensity of the cytosol fraction, while the high-molecular weight pellet was collected and treated for 30 minutes at 37°C with 500 µL of SMM buffer containing 50 µg/mL lysostaphin pre-warmed to 37°C. The mixture was then centrifuged at 14000 rpm for one minute, and the supernatant was removed and placed on ice. 500 µL of 10% sodium dodecyl sulfate (SDS) was added to the cold supernatant mixtures. 100 µL of each sample was taken, and fluorescence intensities were determined in triplicate using a Tecan Platereader (em 485 nm, ex 535 nm). Gain was set to the intensity of the wild type bacterial cell wall that had been incubated with peptide.

Cell wall fractionation data were derived by first subtracting the appropriate blank (cell wall or cytosol) from fluorescence intensity values, then measuring the difference between this value and the fluorescence reading obtained for control conditions (i.e., wild-type bacteria not treated

with peptide). Entries represent means from triplicate experiments and error bars indicate standard deviations.

Preparation of Cells for Electron Microscopy

After following the generic proceure above, labeled cells were resuspended in 4% paraformaldehyde in PBS and incubated at room temperature on a nutating mixer for 45 minutes. Samples were then pelleted, washed three additional times with PBS, and then suspended in 5% gelatin. Suspensions were trimmed to small blocks and placed in 2.3M sucrose solution (cryoprotectant) overnight on a rotor at 40 ^{o}C. They were transferred to aluminum pins and frozen rapidly in liquid nitrogen. The frozen block was trimmed on a Leica CryoUltraCut and 75 nm thick sections were collected using the Tokoyasu method.[4] The frozen sections were collected on a drop of sucrose, thawed, placed on a nickel formvar/carbon coated grid, and floated in a dish of PBS ready for immunolabeling.

For immunolabeling, grids were placed on drops of 0.1 M ammonium chloride for 10 minutes to quench untreated aldehyde groups, then blocked for nonspecific binding on 1% fish skin gelatin for 20 mins. Grids were incubated on primary antibody (rabbit anti-FITC IgG, Invitrogen) at 1:10 and 1:50 dilutions for 30 min. Controls were also performed using Rabbit IgG (Jackson) at the same dilution. Samples were then rinsed 6 times on drops of PBS, and labeled using 12 nm anti-rabbit colloidal gold (Jackson) for 30

minutes. Grids were rinsed in PBS and fixed using 1% gluteraldehyde for 5 min, and a final rinse step with distilled water was performed before transfering grids to a UA/methylcellulose drop for 10 min. Grids were dried and viewed in a FEI Tencai Biotwin TEM at 80Kv. Images were acquired using Morada CCD and iTEM (Olympus) software.

MALDI-TOF Mass Spectroscopy Experiments

Following the generic protocol for labeling above, bacteria were diluted with 3.5 mL of PBS and subjected to ultrasonication using a Branson Sonifier 450 (three one minute sessions at 80% duty cycle, 4/10 output). The pellet was then subjected to enzymatic digest with mutanolysin (150 μg/mL) and lysostaphin (100 μg/mL) in 500 μL of Tris-HCl buffer (0.5 mM, pH 6.5). After gentle mixing, the solution was incubated at 37°C for 4 hours. Samples were then pelleted, and the supernatant was isolated and heated at 90 ^{o}C for 5 minutes to inactivate any residual mutanolysin and/or lysostaphin.

Biotinylated peptides were isolated from the supernatant using an Avidin cartridge (Part no. 4326746 Applied Biosystems Inc. (ABI)) with the ABI syringe setup and ABI protocols. Briefly, the cartridge was activated by washing with 2 mL 0.4% trifluoracetic acid, 30% acetonitrile, followed by 2 mL of phosphate buffer pH 7.2 (loading buffer). 300 μL of the sample was diluted to 500 μL using the loading buffer prior to loading onto the Avidin cartridge. The sample was loaded onto the cartridge at a rate of 1 drop/sec

followed with 500 µL of the loading buffer. Additional washing was done using 1 mL of ABI wash buffer 1 (phosphate buffer pH 7.2) and 1 mL of ABI wash buffer 2 (bicarbonate/methanol buffer pH 8.3). The biotinylated peptides were eluted with 800 µL 0.4% trifluoracetic acid, 30% acetonitrile.

The avidin-eluted biotinylated peptides were dried in a Savant SVC100 SpeedVac, prior to dissolving in 4 µL of matrix solvent (0.1% trifluoroacetic acid, 50 % acetonitrile/water). Matrix was prepared by dissolving alpha-cyano-4-hydroxycinnamic acid (CHCA) in the matrix solvent to a concentration of 3.0 mg/mL. Bradykinin was added to the matrix as an internal calibrant at a final concentration of 1.67 fmol/µL. Spotting mixtures were prepared by mixing 1 µL of sample with 4 µL of matrix in an Eppendorf tube. 0.8 µL of each mixture was spotted on a 384 well sample target plate and allowed to air dry. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) was acquired on an Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF Analyzer in reflector positive mode. The mass range from 700-4000 m/z was screened, and the spectra were internally calibrated to the bradykinin standard (protonated, monoisotopic mass of 1060.569 Da).

Cell Surface Labeling with DIFO Reagents

Following the generic protocol for labeling above, washed bacteria were resuspended in ice-cold PBS (500 mL). Click-reagent **7** was then added to

the suspended bacteria to a final concentration of 10 μM, and samples were incubated for 48 hours at 4ºC on a nutating mixer. Cells were then washed three times with PBS and fixed with 4% PFA for 30 minutes. Following three additional washes, fluorescence was analyzed by flow cytometry and epifluorescence microscopy, as described in the generic protocol above.

References

(1) Duthie, E. S.; Lorenz, L. L. *J. Gen. Microbiol.,* **1952**, *6*, 95–107.
(2) Mazmanian, S. K.; Liu, G.; Jensen, E. R.; Lenoy, E.; Schneewind, O. *P Natl Acad Sci Usa,* **2000**, *97*, 5510–5515.
(3) Patel, A. H.; Foster, T. J.; Pattee, P. A. *J. Gen. Microbiol.,* **1989**, *135*, 1799–1807.
(4) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.,* **2008**, *130*, 11486–93.
(5) "The AlexaFluor Dye Series." Molecular Probes, Inc.. 2006-04-06. http://probes.invitrogen.com/handbook/boxes/0442.html. Retrieved 2010-05-18.